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Using ECIS (electric cell-substrate impedance sensing) to monitor the impedance of vertebrate cell monolayers provides a sensitive measure of toxicity for a wide range of chemical toxicants. One major limitation to using a cell-based sensor for chemical toxicant detection in the field is the difficulty in maintaining cell viability over extended periods of time prior to use. This research was performed to identify cell lines suitable for ECIS-based toxicity sensing under field conditions. A variety of invertebrate and vertebrate cell lines were screened for their abilities to be stored for extended periods of time on an enclosed fluidic biochip with minimal maintenance. Three of the ten cell lines screened exhibited favorable portability characteristics on the biochips. Interestingly, all three cell lines were derived from ectothermic vertebrates, and the storage temperature that allowed long-term cell survival on the enclosed fluidic biochips was also at the lower end of reported body temperature for the organism. Future work with the ectothermic vertebrate cells will characterize their sensitivity to a wide range of chemical toxicants.

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Electric cell-substrate impedance sensing, ECIS, Cell monolayer storage, Biochip, Biosensor, Toxicity sensor

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Suitability of invertebrate and vertebrate cells in a portable impedance-based toxicity sensor: Temperature mediated impacts on long-term survival

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ABSTRACT

Using ECIS (electric cell-substrate impedance sensing) to monitor the impedance of vertebrate cell monolayers provides a sensitive measure of toxicity for a wide range of chemical toxicants. One major limitation to using a cell-based sensor for chemical toxicant detection in the field is the difficulty in maintaining cell viability over extended periods of time prior to use. This research was performed to identify cell lines suitable for ECIS-based toxicity sensing under field conditions. A variety of invertebrate and vertebrate cell lines were screened for their abilities to be stored for extended periods of time on an enclosed fluidic biochip with minimal maintenance. Three of the ten cell lines screened exhibited favorable portability characteristics on the biochips. Interestingly, all three cell lines were derived from ectothermic vertebrates, and the storage temperature that allowed long-term cell survival on the enclosed fluidic biochips was also at the lower end of reported body temperature for the organism, suggesting that reduced cellular metabolism may be essential for longterm survival on the biochip. Future work with the ectothermic vertebrate cells will characterize their sensitivity to a wide range of chemical toxicants to determine if they are good candidates for use in a field portable toxicity sensor.

1. Introduction

The ability to protect drinking water from the threat of accidental or intentional chemical exposures is becoming a growing need in both the military and civilian sectors. A wide array of analyte-specific assays exists for detection of chemicals in water (Kelly et al., 2008; Pancrazio et al., 1999; Reardon et al., 2009; States et al., 2003), but a large number of assays would be needed to detect all possible chemicals, and unknown toxic chemicals would still escape detection. To meet this growing need, a variety of toxicity sensors have been developed that can detect a broad range of chemical contaminants (Curtis et al., 2009a; Eltzov and Marks, 2010; Iuga et al., 2009, and O'Shaughnessy et al., 2004). One toxicity sensor that shows great potential for low cost, maintenance-free detection of many chemical contaminants is ECIS sensing of vertebrate cells, which was first described by Giaever and Keese (1992), and further developed by Curtis et al. (2009a,b), Brennan et al. (2012). A change in cellular impedance has been shown to be a sensitive rapid indicator of viability and cytotoxicity (Giaever and Keese, 1993; Keese et al., 1998; Xing et al., 2005, 2006), and thus is appropriate for toxicity sensing of drinking water.

One major challenge with using mammalian cells as toxicity sensors is the inability to maintain cell viability under field conditions for extended periods of times until the sensors are used for water testing. Mammalian cells generally require frequent feedings, a 37 °C heated environment, and exogenously supplied CO_2. A portable cell maintenance system was developed to support mammalian cell health on the ECIS sensors in field conditions, but the system had limitations including high cost, large size, and sufficient consumables for only 9 days of storage time (Curtis et al., 2009b). Even though some mammalian cells may have useful toxicity response characteristics, they are more difficult to maintain, which has hampered the development of field-usable cell-based toxicity sensors (DeBusschere and Kovacs, 2001; Pancrazio et al., 2003, 2004).

Utilizing cell lines from non-mammalians is a potential way to circumvent the maintenance issues arising from using mammalian cells. The recent use of the RTgill-W1 cell line, which is derived from the rainbow trout gill (Bols et al., 1994), illustrates this (Brennan et al., 2012). RTgill-W1 cells are able to evaluate the toxicity of a wide range of chemicals in less than an hour, and remarkably, the

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cells remained viable for 78 weeks without changing the medium of the biochips (Brennan et al., 2012). Thus using RTgill-W1 cells greatly extended the storage capabilities of the ECIS-based toxicity sensor over using mammalian cell lines. Rainbow trout cell lines, however, become stressed at 26 °C and die rapidly at 30 °C (Bols et al., 1992). This may limit the utility of this cell line under certain field conditions.

In an effort to broaden the field capabilities of the ECIS-based toxicity sensor, additional cell lines were examined for their stability on the biochips. Ten cell lines from insects, fish, amphibians, reptiles, and mammals were evaluated in this sensor for their ability to remain as confluent monolayers on fluidic biochips under a broad range of temperatures without media changes or exogenously supplied CO_2. Two cell lines from reptiles and one from fish were identified as superior because they increased the thermal tolerance of the sensor.

2. Materials and methods

2.1. Tissue culture

Ten cell lines were evaluated in the current study. Table 1 lists the cell lines and culture conditions. All cell lines chosen have been characterized fully in published literature, except for the newly isolated WECF11e cells that are described in this paper for the first time.

2.2. Isolation and characterization of WECF11e cells

The epithelial-like cell line that is designated WECF11e (Wall Eye Caudal Fin (fish #11) Epithelial) was derived from a caudal fin of a juvenile walleye (*Sander vitreus*). The walleye (provided by Dr. J.S. Lumsden (University of Guelph)) was from the White Lake Fish Culture Station, Ontario Ministry of Natural Resources, ON, Canada. The original fish stocks were from Lake Ontario, ON, Canada. Primary cultures were established by explant outgrowth of fin fragments. The growth medium was Leibovitz's L-15 medium (L-15; Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO), 200 U/mL penicillin (Thermo Scientific HyClone, Logan, UT) and 200 mg/mL streptomycin (Thermo Scientific HyClone, Logan, UT). Briefly, the caudal fin was removed from an anesthetized fish and excised into small pieces. These fin fragments were rinsed three times with Dulbecco's Phosphate Buffered Saline (DPBS) before being plated in 25 cm^2 culture flasks. Over several weeks cells migrated out from the fragments onto the plastic tissue culture surface. These primary cultures were composed of both fibroblastic and epithelial-like cells, with the latter type being more predominant. When the flasks were confluent, the cells were passaged. With continued growth and passaging of the cultures at 20 °C in L-15 with 5% FBS, nearly all the cells of the culture had an epithelial-like morphology. WECF11e proliferated in cultures incubated at temperatures from 14–32 °C (data not shown); in this regard they were similar to cell lines developed from walleye in the

Table 1
Cell lines used and culture conditions tested.

Cell line designation: tissue origin and species name	Source & reference for each cell line	Media[b]	Temperature (°C)	CO_2
SF9: ovary cells from *Spodoptera frugiperda*	ATCC[a] CRL-1711 (Vaughn et al., 1977)	Grace's medium with 10% heat inactivated FBS (Mediatech Inc., VA)	28	No
HvAM1: pupal ovary cells from *Heliothis virescens*	Dr. C Goodman, USDA (McIntosh and Ignoffo, 1983)	Ex-cell 420 medium (Sigma-Aldrich, MO) with 10% heat inactivated FBS	Room temperature[c]	No
Sua1B: larval cells from*Anopheles gambiae*	Dr. J Bloomquist, U of Florida (Dimopoulos et al., 1997)	Schneider's Drosophila medium (Life Technologies, NY) with 10% heat inactivated FBS	Room temperature	No
S2: embryonic cells from *Drosophilia melanogaster*	R690-07 (Life Technologies, NY) (Schneider, 1972)	Schneider's Drosophila medium (Life Technologies, NY) with 10% heat inactivated FBS	28	No
FHM: connective tissue/muscle epithelial cells from *Pimephales promelas*	ATCC CCL-42 (Gravell and Malsberger, 1965)	Eagle's minimum essential medium (EMEM) in Hanks' BSS (Life Technologies, NY) with 10% FBS	Room temperature	No
ICR134: embryonic epithelial cells from *Rana pipiens*	ATCC CCL-128 (Freed and Mezger-Freed, 1970)	50% Leibovitz's L-15 medium, 40% distilled water, and 10% FBS	Room temperature	No
GL-1: lung epithelial cells from *Gekko gecko*	ATCC CCL-111 (Cohen and Clark, 1968)	Adapted to grow in Leibovitz's L-15 medium with 15% FBS	Room temperature	No
IgH-2: heart epithelial cells from *Iguana iguana*	ATCC CCL-108 (Clark et al., 1970)	Adapted to grow in Leibovitz's L-15 medium with 10% FBS	Room temperature	No
WECF11e: fin epithelial cells from *Sander vitreus*	Dr. N. Bols, U of Waterloo described here	Leibovitz's L-15 medium with 5% FBS	26	No
15P-1: Sertoli cells from *Mus musculus*	ATCC CRL-2618 (Paquis-Flucklinger et al., 1993)	Adapted to grow in Leibovitz's L-15 medium with 10% FBS	32[d]	No

[a] American type culture collection (ATCC; Manassas, VA).
[b] All media purchased from Lonza (Walkersville, MD) unless otherwise noted.
[c] Room temperature is 25 °C.
[d] Can also be grown at room temperature.

20th century but are no longer available (Kelly et al., 1980; Wolf and Mann, 1980). Monolayer cultures of WECF11e were stable (little change in morphology and no cell detachment) when incubated at 4 °C for over 3 months without medium changes. WECF11e has been passaged over 30 times and been cryopreserved successfully. These features made WECF11e a promising candidate for use in the ECIS-based toxicity sensor.

2.3. Cell seeding and storage on open well ECIS chips

A variety of adhesion substrates were evaluated with each cell line to determine the preferred substrate (Table 2) that would facilitate uniform cell attachment to the gold electrodes on open well ECIS chips (#8W10E; Applied BioPhysics, Troy, NY). Adhesion substrates were prepared according to manufacturers' instructions. Prior to being seeded with cells, 200 μl of one of the substrate solutions [laminin (50 μg/ml), fibronectin (50 μg/ml), collagen I (50 μg/ml), collagen IV (50 μg/ml), poly-D-lysine (50-200 μg/ml), gelatin (0.2% solution), or concanavalin A (0.8-3.2 mg/ml)] was added to the wells of each chip, incubated for 1–2 h, and washed with appropriate growth medium.

Each cell line was seeded at a specific cell density (FHM cells at 6×10^5 cells/well and all other cell lines at 1 or 2×10^5 cells/well), with media replacements done 3× a week. The seeded chips were stored for a two-week period at the culture temperatures indicated in Table 1, during which time the impedances were recorded as indications of the cellular monolayer integrity. A cell monolayer was determined to be stable over the two-week storage period if the impedance values did not decrease more than 20%, and if the morphology of the cells did not change substantially as visualized by phase contrast microscopy. The 20% reduction in impedance was defined as the limit to acceptable reduction based on previous experiments using confluent cell monolayers for toxicity testing (Curtis et al., 2009a,b; Brennan et al., 2012). For the system to be sensitive to chemical toxicity, a significant separation in impedance between the healthy and affected cells needs to be established and maintained. Cell lines that formed stable monolayers for two weeks on the open well ECIS chips were then selected for seeding in the enclosed fluidic biochips, which are used for the field-portable ECIS-based toxicity sensor.

2.4. Cell seeding and storage on the enclosed fluidic biochip

Sterile fluidic biochips used in this study were purchased from Applied BioPhysics (Troy, NY). The fluidic biochips were made from two components; an upper polycarbonate layer with two separate fluidic channels (manufactured by Biosentinel Inc., Austin, TX), and a lower electronic layer that contained four electrode pads (each electrode pad contained ten 250 μm diameter electrodes) per channel). Fluidic biochip design information is described in detail by Curtis et al. (2009b). Fig. 1 shows a picture of a fluidic biochip seeded with cells and enclosed with sterile PharMed® BPT biocompatible tubing (Saint-Gobain Performance Plastics, Akron, OH).

For cell testing on the fluidic biochips, the chips were first coated with selected adhesion substrates. The preferred substrate for each cell line is listed in Table 2, and this was previously determined by screening the cells on multiple substrates during the ECIS open well experiments. Cells were seeded at densities of $2–5 \times 10^5$ cells/ml, depending upon the cell type. Sterile PharMed® BPT was used to form closed loops on the ends of the biochips. The seeded biochips were stored at the optimal growing temperature for the cells (listed in Table 1), and fed with growing medium 3× a week over a 1–2 week initial feeding period to allow the cells to form a confluent monolayer inside the biochip. The chips were then placed in different temperature environments to examine long-term (>90 days) cell viability. The cells in the fluidic biochip were not fed during the maintenance period. Viability of the monolayer was determined by monitoring impedance values over the storage time. Different temperature environments were created by using a lab refrigerator (6 °C), a Fisher Scientific Isotemp refrigerated incubator (20 °C), and a Barnstead Thermolyne Type 37900 culture incubator (33 °C, 36 °C, and 37 °C). The 25 °C degree environment was the average daily temperature in the laboratory.

The GL-1 cells (ATCC #CCL-111) were originally isolated from *Gekko gecko* (Cohen and Clark, 1968). The optimal core body temperature of this lizard is 26.8–27.8 °C, with a tolerable range of 18.2–35.6 °C (Stokes and Meek, 2003). The GL-1 cells seeded in the biochips were incubated at 6, 25, or 33 °C to determine the optimal environmental temperature for cell storage.

The IgH-2 cells (ATCC #CCL-108) were originally isolated from *Iguana iguana* (Clark et al., 1970). The organism has a range of documented core body temperatures from 24.5–43.1 °C (van Marken Lichtenbelt et al., 1997). The IgH-2 cells seeded in the biochips were incubated at 6, 20, 25, or 36 °C to determine the optimal environmental temperature for cell storage.

The WECF11e cells were isolated from *Sander vitreus* (walleye; isolation described here). With walleye, the upper incipient lethal temperature is 34 °C for sub-yearling fish (Wilson and Nagler, 2006). The lower incipient lethal temperature is less certain, 6 °C has been suggested (Hokanson, 1977), but spawning occurs between 3 and 10 °C (Wismer and Christie, 1987). The WECF11e cells seeded in the biochips were incubated at 6, 20, 25, or 37 °C to determine the optimal environmental temperature for cell storage.

Table 2
Suitability of cell lines for use in the ECIS based biosensor.

Animal group, cell line designation & animal origin (common name)		Preferred substrate[a]	Formation of stable monolayer yielding a high impedance (>1000 ohms)[b]	Promise in a portable biosensor
Invertebrate cells	**SF9**: fall armyworm	Concanavalin A	No	No
	HvAM1: tobacco budworm	Concanavalin A	No	No
	Sua1B: mosquito	Concanavalin A	No	No
	S2: fruit fly	Concanavalin A	No	No
Vertebrate non-mammalian cells	**FHM**: fathead minnow	Collagen I	Yes	No[c]
	ICR134: northern leopard frog	Fibronectin	No	No
	GL-1: gecko	Collagen I	Yes	Yes
	IgH-2: green iguana	Fibronectin	Yes[d]	Yes
	WECF11e: walleye	Gelatin	Yes	Yes
Mammalian cells	**15P-1**: mouse	Fibronectin	Yes	No

[a] All adhesion substrates purchased from Sigma-Aldrich (St. Louis, MO) except fibronectin and collagen I, which were purchased from Calbiochem (San Diego, CA).
[b] Formation of stable monolayer in this report is defined as less than a 20% reduction in impedance values over a 2-week storage time in open well ECIS chips.
[c] Variable impedance readings.
[d] Stable impedance readings over 1000 ohms only achieved at 20 °C storage temperature.

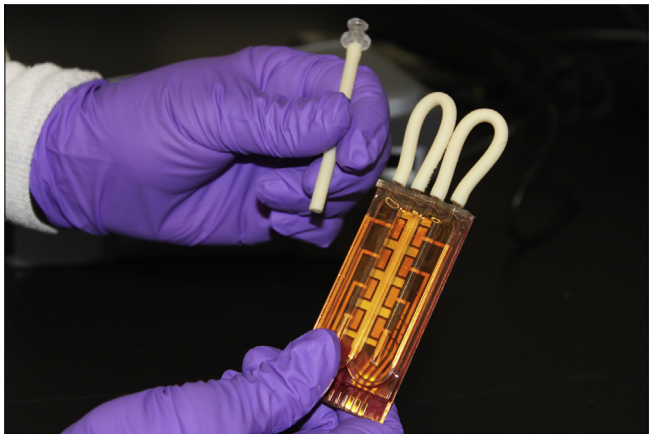

Fig. 1. Fluidic biochip enclosed with tubing. The biochip is seeded with cells using tubing shown and stored for extended periods of time without medium replacement at desired temperature and with no exogenously-supplied CO_2.

2.5. Impedance measurement with ECIS

Impedance measurements of cells on the open well chips and fluidic biochips were monitored using the ECIS 1600 analyzer (Applied BioPhysics, Troy, NY) as previously described by Curtis et al. (2009a).

3. Results

3.1. Cell storage in open well ECIS chips

Table 2 summarizes the cells performance in open well ECIS chips. None of the invertebrate cells could form a monolayer that yielded impedance values greater than 1000 ohms. The HvAM1 cells had the highest impedance values (900 ohms) of all the invertebrate cell lines tested, but the cell monolayer became clumpy over the 2-week period, causing the impedance values to drop. The adhesion substrates fibronectin and poly-D-lysine were also evaluated with the invertebrate cells, but for all four invertebrate cell lines the concanavalin A (lectin extracted from the jack bean) supported the strongest cell attachment and highest impedance readings.

The five vertebrate non-mammalian cell lines were each chosen because of their ability to be maintained at different temperatures and their lack of requirements for exogenously supplied CO_2 for cell growth and maintenance (detailed in Table 1). As summarized in Table 2, all the vertebrate cell lines formed a stable monolayer on the open well ECIS electrodes yielding high impedance values, except the ICR134 cell line from *Rana pipiens*. These cells initially formed a stable high impedance monolayer, but because they were not contact inhibited, the cells began to layer on top of one another, which resulted in decreased impedance. The IgH-2 cell line from *Iguana iguana* was not stable for 2 weeks in the open well chips when incubated at the ATCC recommended growing temperature of 25 °C, but was stable at an incubation temperature of 20 °C.

The 15P-1 cell line, (Sertoli cells isolated from the testis of *Mus musculus*) was the one mammalian cell line included in this study because of its ability to be stored at temperatures other than 37 °C. The 15P-1 cells are normally grown in Dulbecco's Modified Eagle's Medium with 10% FBS in a 5% CO_2 environment. To reduce the storage requirements of the 15P-1 cell line, the cells were slowly adapted to Leibovitz's L-15 with 10% FBS, which is a CO_2 independent medium. The 15P-1 cells were stable for 2 weeks at the optimal growth temperature (32 °C) and also at room temperature without the addition of exogenously added CO_2. Cell lines that could form stable confluent monolayers on the open wells were then evaluated for long-term survival on the enclosed fluidic biochip.

3.2. Cell storage in enclosed fluidic biochip

Of the five cell lines screened in the fluidic biochip (FHM, GL-1, IgH-2, WECF11e, and 15P-1), only three of the cell lines could form stable monolayers (GL-1, IgH-2, and WECF11e). The FHM cells survived in the fluidic biochip for 90 days at temperatures of 12, 20 and 25 °C, but the impedance readings were highly variable as illustrated by the large standard error of mean (SEM) values (Fig. 2). The variability in impedance values during storage makes the FHM cells unsuitable for consistent toxicity detection. The 15P-1 cells were an unusual mammalian cell line because they could be stored at room temperature with no exogenously added CO_2, and they had impedance values above 1000 ohms. There was a 20% reduction in impedance values, however, over the two week measurement period, which lessens the likelihood of long-term cell viability and consistent toxicity detection. The three cell lines that showed promise in the fluidic biochip were tested further to characterize the stability of the monolayers over a range of storage temperatures.

Long-term (>90 days) storage of cells in the enclosed fluidic biochip, with no gas or medium changes, has special challenges including lack of oxygen and build-up of CO_2 and other cellular waste products that can impact cell health. Both lactate and ammonia accumulation has been shown to limit cell health in long-term cultures (Hassell et al., 1991). To optimize the longevity of the cells in the enclosed fluidic biochip without media replenishment for an extended period of time, we specifically chose to use Leibovitz's L-15 (Lonza, Carlsbad, CA) without L-glutamine. This medium was chosen for long-term storage of cells because galactose is used as an energy source instead of glucose. Galactose is preferable because it has been shown to decrease the production of lactate, and thus slow the drop in medium pH over time (Altamirano et al., 2000). This medium was free of L-glutamine, and was supplemented with GlutaMAX (Life Technologies, NY) which has been shown to decrease ammonia production in long-term cultures.

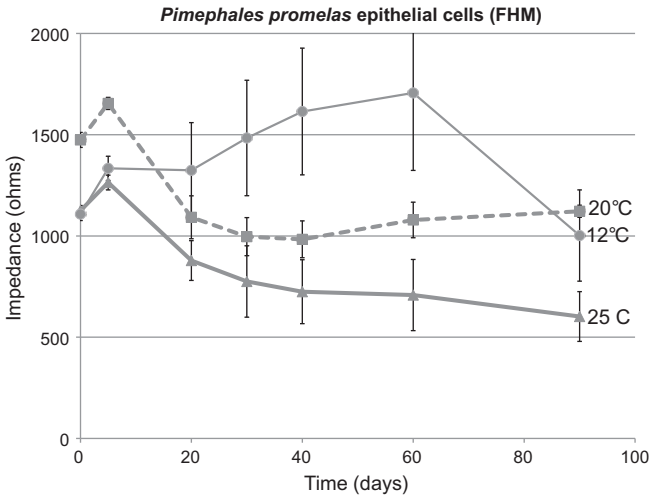

Fig. 2. Impedance readings of FHM cells stored in fluidic biochips at different temperatures. The impedance data is represented as the mean ± SEM of 8 separate electrodes on a fluidic biochip.

Fig. 3 shows the impedance readings of the *Gekko gecko* lung epithelial cells (GL-1), *Iguana iguana* heart epithelial cells (IgH-2), and the *Sander vitreus* fin epithelial cells (WECF11e) over at least a 90 day period in enclosed fluidic biochips stored at different temperatures. The impedance data shows that each cell line has a preferred storage temperature that allows the cells to be stored long-term in the enclosed fluidic biochip with no medium replenishment. The GL-1 cells displayed stable impedance readings over the measurement period when stored at 25 °C; however, when the cells were stored at 6 or 33 °C there was a quick drop in impedance readings. The IgH-2 cells displayed stable impedance readings over the measurement period when stored at 20 °C; however, when the cells were stored at 6 or 36 °C there was a quick drop in impedance readings. IgH-2 cells stored at 25 °C were stable for 10 days before impedance values began decreasing. The WECF11e cells displayed stable impedance readings over the measurement period when stored at 6 °C. Storage of the cells at 37 °C caused a quick decrease in impedance values; whereas storage at 20 °C or 25 °C caused a slow decrease in impedance values over the maintenance period.

4. Discussion

Interestingly, for all three cell lines that could be stored on the fluidic chip, the optimal long-term maintenance temperature was at the lower end of the organism's core temperature. This data suggests that a reptilian cell line stored at 20 °C or 25 °C, and a cold water fish cell line stored at 6 °C are able to survive in the enclosed biochip environment with no medium replenishment because the metabolism of the cells is low, which would result in a decrease in the production of waste products that can negatively impact cell health. Ectothermic animals, unlike endothermic animals, have a fluctuating body temperature and metabolism based on the environmental temperature. Thus, at low environmental temperatures, the ectothermic animal has very little energy requirements and is in a hypometabolic state. The relationship between metabolism and temperature in the ectothermic cell line makes it an ideal candidate for field portable sensors. The cells can be very easily stored at low temperatures in a hypometabolic state, and then the temperature can be raised when the cells are required for toxicant testing.

It was determined in this study that each cell line has very specific temperature requirements to allow long-term storage on an enclosed biochip. From the data presented here, it seems likely that other cell lines isolated from ectothermic animals will store well in the enclosed fluidic biochip at temperatures in the low end of the organism's range. Other cell lines from the rainbow trout (RTgutGC (Kawano et al., 2010) and RTL-W1 (Lee et al., 1993)), which are derived from the intestine and liver, respectively, have been shown to survive for 9 months on the fluidic biochip at 6 °C with no media replenishment, and still respond to toxicant challenges using ECIS (unpublished data, USACHER, 2012). This data supports the idea that different cells from the same organism (in this case the rainbow trout) have storage temperatures that are similar and in alignment with the whole organism's core temperature. This type of information may help facilitate the selection of cell lines for specific testing platforms and environments. Currently, the rainbow trout gill epithelial cells (RTgill-W1) are the preferred cell line in the ECIS-based toxicity sensor because of refrigerated storage (6 °C) capabilities and chemical toxicant detection (Brennan et al., 2012). Exploration of other candidate cell lines will allow the ECIS-based toxicity sensor to have flexibility in the field. If 6 °C storage is not available, then cell lines with different optimal storage temperatures could be utilized and tailored for the testing environment. It has been shown that fish and reptilian cell lines have toxicant sensitivities comparable to mammalian cell lines

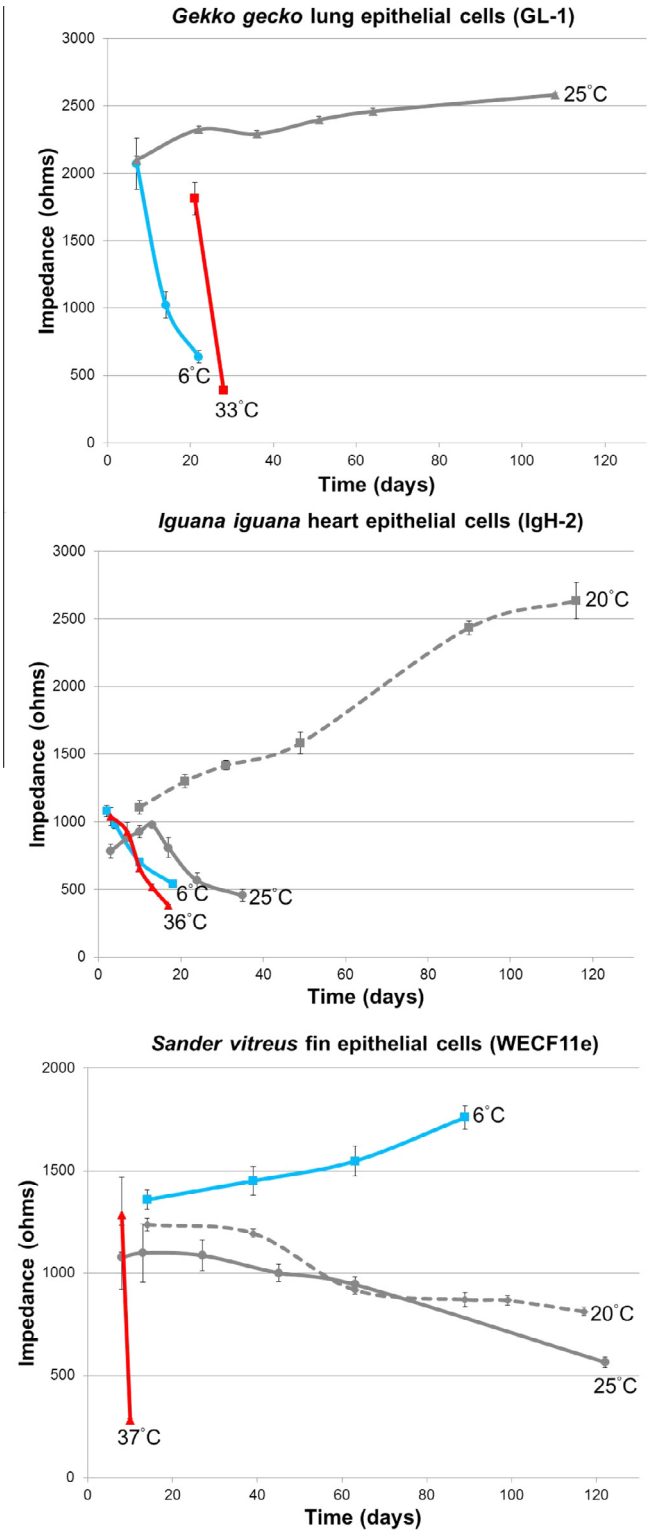

Fig. 3. Impedance readings of cells stored in fluidic biochips at different temperatures. The impedance data is represented as the mean ± SEM of 8 separate electrodes on a fluidic biochip.

(Brennan et al., 2012; Curtis et al., 2009a); indicating that a wide variety of vertebrate cells could be used as accurate predictors of human toxicity.

Future planned research will determine the chemical toxicant sensitivity and response time of the GL-1, IgH-2, and WECF11e cells using ECIS. These cells may increase the range of chemicals

currently detected by the RTgill-W1 cells, which would add value to the ECIS-based toxicity sensor.

Conflict of interest

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.07.007.

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